

**PATENT****IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re: Patent application of  
Mortimer Civan et al.

Serial No.: 10/009,581

Filed: April 30, 2002

For: Methods for Controlling IntraOcular Pressure

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: Group Art Unit: 1614  
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: Examiner: Donna Jagoe  
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: Conf. No. 1751  
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**DECLARATION OF DR. MORTIMER M. CIVAN under 37 C.F.R. § 1.132**

**Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450**

**Sir:**

I, Mortimer M. Civan, declare that:

1. I am an inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 10/009,581, claiming for the first time that it is possible to regulate intraocular pressure in an eye by administering to the eye a pharmaceutical composition comprising a pressure-modulating amount of a sodium-hydrogen exchanger (NHE) inhibitor.

2. To facilitate submission of this Declaration, I have not attached a copy of my *curriculum vitae*. However, should my c.v. become required, I will produce it upon request. Briefly, however, my career has been devoted to studies of the basis for the formation of aqueous humor in the eye in order to better understand and treat clinical glaucoma. I am currently Professor of Physiology and Medicine, Department of Physiology at the University of Pennsylvania School of Medicine, and a Member of the Cell and Molecular Biology Graduate Group. I hold an M.D. degree from Columbia University, NY and an A.B. *summa cum laude* from Columbia College, NY with a major in chemistry. I have been honored with several awards, including being elected a Fellow of American Association for the Advancement of Science, selected as an Established Investigator for the American Heart Association, a Member of the American Society for Clinical Investigation, selected as Overseas Fellow at Churchill College, University of Cambridge, a Faculty Scholar of the Josiah Macy, Jr. Foundation,

recipient of the Dean's Award for Excellence in Teaching Basic Science, University of Pennsylvania School of Medicine, and appointed Harold Chaffer Memorial Lecturer on the Faculty of Medicine, University of Otago, Dunedin, New Zealand. In addition, I was editor of *The Eyes' Aqueous Humor: From Secretion to Glaucoma*, Academic Press, San Diego, CA, 1998, and have been invited by Elsevier Press to edit a new edition of that work.

3. I have read all of the communications from the U.S. Patent and Trademark Office (PTO) relating to the above-identified application, and I have participated in the Response process for each Office Action. I was also an active participant at the in-person Examiner's Interview for this application on October 27, 2005 and spoke with Examiner Jagoe at length on the subject of my invention and the comments made in the Office Action dated August 11, 2005. I am well qualified to discuss NHE inhibitors and beta blockers in regard to my claimed invention.

4. Claims 94-106 are pending in our application. Claim 94 is an independent claim, and all other pending claims are directly or indirectly dependent upon claim 94. Claim 94 reads as follows:

94. A method of regulating intraocular pressure by inhibiting sodium/hydrogen antiport activity in the eye, comprising administering to ciliary epithelial cells in an eye of a human or an animal having a trabecular network a pharmaceutical composition, wherein the pharmaceutical composition comprises a pressure-modulating amount of at least one sodium-hydrogen exchanger (NHE) inhibitor.

5. The class of molecules designated "NHE inhibitors" are known in the art. They have been, and continue to be, used in cardiovascular therapies. At the time of the invention, NHE inhibitors did not include timolol or any other beta blocker. For instance, Karmazyn discloses a variety of NHE inhibitors (*Ann. N.Y. Acad. Sci.* 874:326-334 (1999)). See pp. 326-327, bridging sentence. Karmazyn does not disclose or suggest timolol or any other beta blockers are NHE inhibitors. Subsequent to the invention, NHE inhibitors still do not include beta blockers. For instance, Masereel *et al.* review numerous NHE inhibitors but do not teach or suggest beta blockers are NHE inhibitors (*Eur. J. Med. Chem.* 38:547-554 (2003)). Thus, the term "NHE inhibitors" does not refer to or suggest beta blockers.

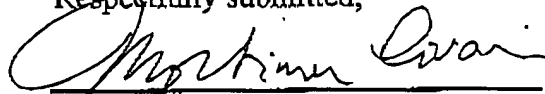
6. In our instant application, timolol is identified properly as a beta blocker. See for instance, p. 3, lines 25 and p. 4, lines 19. Therefore, claim 94 does not embrace timolol or any

other beta blockers. There is no definitive scientific proof that timolol acts on the NHE antiport of the eye.

7. Our claimed invention is one in which administration of a pressure-modulating amount of an NHE inhibitor to an eye, having a trabecular network, regulates intraocular pressure. No one in the art demonstrated or knew, before my present invention, that the antiport inhibitors *per se* lower intraocular pressure, and further control the uptake and release of salts within the aqueous humor of the eye. As a result the methods drawn to the use of the NHE inhibitors in our invention are, in fact, both novel and nonobvious.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,

  
Mortimer M. Civan

Date: November 9, 2005

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P. 1/4

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# The Role of the Myocardial Sodium-Hydrogen Exchanger in Mediating Ischemic and Reperfusion Injury

## From Amiloride to Cariporide<sup>a</sup>

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**ABSTRACT:** There is convincing evidence that the Na-H exchanger (NHE) plays a pivotal role in mediating tissue injury during ischemia and reperfusion. Extensive studies with NHE inhibitors have consistently shown protective effects against ischemic and reperfusion injury in a large variety of experimental models and animal species, particularly in terms of attenuating contractile dysfunction. These protective effects of NHE inhibition appear to be superior to other strategies, including ischemic preconditioning. Such studies have contributed greatly to the overwhelming evidence that NHE activation mediates ischemic and reperfusion injury. The NHE inhibitor HOE 642 (cariporide) is currently undergoing clinical evaluation in high-risk cardiac patients. Moreover, there is now emerging evidence that NHE may be involved in mediating cardiotoxicity directly produced by various ischemic metabolites such as lipid amphiphiles or reactive oxygen species. NHE inhibition also attenuates apoptosis in the ischemic myocardium, a process that may be of importance in the subsequent development of postinfarction heart failure. In conclusion, NHE represents an important adaptive process in response to intracellular acidosis that results in a paradoxical contribution to cardiac tissue injury.

### NHE AND THE ISCHEMIC MYOCARDIUM: A BRIEF HISTORICAL PERSPECTIVE

NHE represents one of the major pH regulatory systems in the cardiac cell. The concept that this system may be involved in cardiac pathology was first proposed by Lazdunski and coworkers in 1984<sup>1</sup> based on the observation that sodium influx concomitant with proton efflux may produce undesirable effects through disordered calcium homeostasis. The first experimental evidence came from the author's laboratory in 1988,<sup>2</sup> where it was reported that amiloride, a relatively non-specific NHE inhibitor, protected the ischemic and reperfused myocardium. Since that initial observation dozens of studies have reported on the protective effects of NHE inhib-

<sup>a</sup>Studies from author's laboratory are supported by the Medical Research Council of Canada. The author is a Career Investigator of the Heart and Stroke Foundation of Ontario.

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itors including more selective amiloride analogues and the more recently developed compounds HOE 694 and HOE 642 (cariporide). It has been particularly impressive that virtually all reports from a variety of laboratories have demonstrated protective effects. These impressive findings formed the basis for the rapid approval and establishment of a multicentered international clinical trial, the GUARDIAN study, to assess the effects of cariporide in high-risk patients with acute coronary syndromes. The four major group of patients recruited into this study include (1) patients with unstable angina, (2) patients with non-Q wave myocardial infarction, (3) unstable patients requiring percutaneous transluminal coronary angioplasty, and (4) unstable or high-risk patients requiring coronary artery bypass surgery. The two primary endpoints are myocardial infarction and mortality. The results of the trial are expected in 1999. Taken together, these developments represent rapid progress in the development of novel strategies in cardiovascular therapeutics that will hopefully result in a reduction in morbidity and mortality in patients with heart disease.

### INTRODUCTION: $\text{pH}_i$ REGULATION AND NHE

Changes in intracellular pH ( $\text{pH}_i$ ) can produce marked effects on cardiac contractility, particularly acidosis-induced negative inotropic effects. Although the mechanisms involved in pH-regulated contractility are very complex, they reflect the direct interfering effects of protons on various cellular processes involved with excitation-contraction coupling.<sup>3</sup> It is therefore critical that the cell possesses mechanisms by which  $\text{pH}_i$  is regulated especially after intracellular acidosis as a consequence of myocardial ischemia. Two major alkalizing exchangers exist in the cardiac cell, the NHE and a  $\text{Na-HCO}_3^-$  symport. The NHE represents one of the key mechanisms for restoring  $\text{pH}_i$  following ischemia-induced acidosis by extruding protons concomitantly with sodium influx. The simultaneous entry of sodium during NHE activation indicates that this process is also an important route for increasing intracellular sodium concentrations during various conditions and represents the major mechanism postulated to mediate NHE-dependent cardiac injury through modulation of intracellular calcium levels, as discussed further below. To date, at least five (a mitochondrial NHE-6 isoform has been identified although its function is unknown) distinct isoforms of the exchanger have thus far been identified that possess structural differences as well as varying sensitivities to inhibition by pharmacological agents. It appears that the ubiquitous subtype 1 (NHE-1), a glycoprotein with a molecular weight of approximately 110 kD, is the predominant isoform in the mammalian myocardium. NHE-1 comprises two major functional domains: a hydrophobic region that spans the membrane 12 times and that is critical for exchange activity, and a hydrophilic moiety that is likely of importance for hormonal modulation of the exchanger. Further details concerning the molecular structure and regulation of the NHE can be found in a number of recent review articles and monographs.<sup>4-6</sup> The activity of NHE-1 can be modulated by a number of growth factors, hormones, and neurotransmitters (via various kinases, including tyrosine kinases, Ca-calmodulin kinase, MAP kinases, and PKC coupled to G proteins), as well as by hypertonic shrinking and mechanical stimuli. Most of these signals stimulate NHE by shifting the  $\text{pH}_i$ -activity curve towards the alkaline range, thus stimulating the enzymatic activity of the transporter at constant  $\text{pH}_i$  and moving

**TABLE 1.** Beneficial effects of Na-H exchange inhibitors on the ischemic and reperfused heart

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Enhanced/accelerated systolic recovery after reperfusion
Diminished ischemic and reperfusion-induced contracture
Reduced arrhythmias including suppression of postinfarction ventricular fibrillation
Prevention of postinfarction mortality
Reduced calcium and sodium overload
Preservation of energy metabolites
Reduced necrosis
Reduced apoptosis
Reduced toxicity of various ischemic metabolites and paracrine/autocrine factors

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it closer to its maximal rate.<sup>7</sup> Conversely, inhibition of the exchanger can be accomplished by a variety of drugs, the prototypical being amiloride and its *N*-5 disubstituted derivatives.<sup>8</sup> Recently, the benzoyl guanidinium compounds 3-methylsulfonyl-4-piperidinobenzoyl-guanidine methanesulfonate (HOE 694) and 4-isopropyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE 642) have been shown to be effective NHE inhibitors. HOE 642 (cariporide) is of particular interest as it appears to be a selective inhibitor of the NHE-1 isoform, the primary if not sole subtype found in heart, rendering it particularly attractive for therapeutic intervention in cardiac disorders while minimizing the potential for side effects.<sup>9</sup>

#### EFFECTS OF NHE INHIBITORS ON THE ISCHEMIC AND REPERFUSED MYOCARDIUM

Evidence in the literature strongly supports the concept that NHE inhibition bestows excellent myocardial protection against ischemic and reperfusion injury. As discussed in a number of recent reviews<sup>6,10-12</sup> and as summarized in TABLE 1, these salutary effects have been demonstrated on numerous parameters of cardiac function, including enhanced contractility, reduced contracture, and a decrease in the incidence of arrhythmias. In addition, improvements in biochemical and ultrastructural indices have been extensively demonstrated with NHE inhibition. Such protection has been demonstrated with the amiloride series of agents and with both HOE 694 and HOE 642. This protection is associated with diminished tissue sodium and calcium content, in support of a close association between NHE and Na-Ca exchange activity.<sup>13,14</sup> Indeed, reduction in calcium overload appears to represent a major mechanism of action of NHE inhibition (see below).

#### INTERACTION BETWEEN NHE INHIBITORS AND OTHER CARDIOPROTECTIVE STRATEGIES

Studies have been done to ascertain potential interactions between NHE inhibitors and other pharmacological agents or approaches used to protect the myocardium. Such interactions are of potential importance as they may be relevant when utilizing multifaceted approaches towards myocardial protection. For example, it

has been shown that amiloride enhanced the protection afforded by reduction of extracellular sodium and calcium concentrations in isolated working rat hearts subjected to 30 min of cardioplegic arrest.<sup>15</sup> Moreover, in isolated working rat hearts subjected to ischemia, administration of amiloride in combination with the hydroxyl radical scavenger desferrioxamine produced superior cardioprotective effects compared to each drug alone.<sup>16</sup> These results are therefore suggestive of a specific and distinct target for the beneficial effects of NHE inhibition rendering this approach attractive for potential superior cardioprotective strategies using drug combination protocols. Moreover, we have recently demonstrated additive protective effects of HOE 642 when the drug is administered in combination with either of the volatile anesthetics sevoflurane or isoflurane in isolated ischemic and reperfused rat hearts.<sup>17</sup> Although indicative of distinct mechanisms of action, these findings also suggest that the combination of HOE 642 and these volatile agents produces superior cardioprotection, which may be of importance under clinical conditions where effective cardioprotection is desired during surgical procedures.

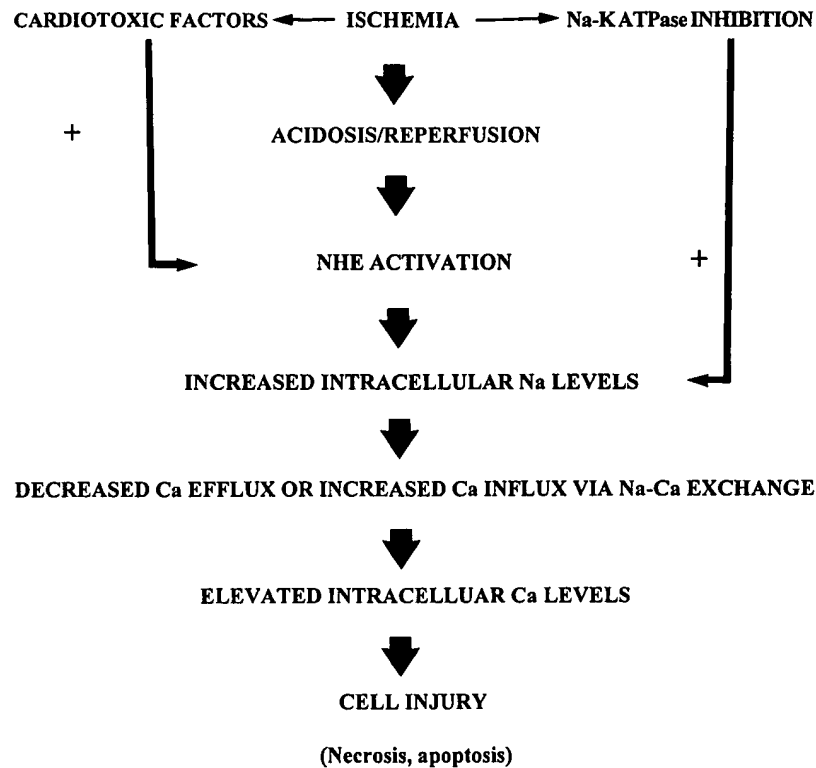
#### **IS NHE INHIBITION A SUPERIOR CARDIOPROTECTIVE STRATEGY? COMPARISON WITH ISCHEMIC PRECONDITIONING**

Detailed comparisons between NHE inhibition and other modes of cardioprotection have not been extensively studied yet these types of studies are important in order to design the most effective therapeutic approaches. Work in our laboratory has compared ischemic preconditioning with NHE inhibition with cariporide in hearts subjected to increasing periods of zero-flow ischemia followed by 30-min reperfusion. Ischemic preconditioning was carried out with two 5-min cycles of ischemia separated by a 10-min reperfusion before initiating prolonged ischemia. Alternatively, experiments were also done in the presence of 5  $\mu$ M cariporide. The essence of our findings is that improved recovery of function is similar with the shorter ischemia durations although cariporide-treated hearts recovered significantly faster. The most marked observation was that cardioprotective effects of cariporide were still evident when ischemia was extended to 90 min, whereas no salutary effects were seen with ischemic preconditioning. Accordingly, it appears that NHE inhibition is advantageous and superior to ischemic preconditioning, particularly under conditions of prolonged ischemia when the effects of the latter are no longer evident.

#### **MECHANISMS OF NHE INVOLVEMENT IN ACUTE ISCHEMIC AND REPERFUSION INJURY**

A number of concepts are emerging regarding the mechanisms underlying NHE involvement in the ischemic myocardium and it appears that NHE may be involved in multiple mechanisms. FIGURE 1 outlines these concepts and shows that these mechanisms are interrelated, suggesting that although calcium is the final mechanism, the activation of NHE and the role of the antiporter in mediating injury likely involve multifaceted aspects.





**FIGURE 1.** Pathways of NHE activation and its involvement in acute responses to myocardial ischemia and reperfusion. See text for details.

#### *Modulation of Intracellular Calcium Levels*

The role of calcium is based on the proposal that reintroduction of flow to the previously ischemic, and thereby acidotic, cardiac cell establishes a rapid transsarcolemmal proton gradient resulting in activation of NHE. This has been reviewed in several publications.<sup>1,6,10-12,14</sup> While this would contribute to restoration of  $\text{pH}_i$ , the concomitant sodium influx could result in increased intracellular calcium concentration via the Na-Ca exchanger due to reduced calcium efflux resulting from the reduction of the sodium gradient driving the Na-Ca exchanger. Moreover, it should be emphasized that in the ischemic cardiac cell Na-K ATPase is inhibited resulting in an elevation in intracellular sodium concentration due to reduced ability to extrude sodium. Activation of NHE, particularly upon reperfusion, which in itself is greater due to the prior ischemia-induced accumulation of protons, provokes a greater elevation in intracellular sodium and calcium concentrations particularly under conditions of defective ion regulatory mechanisms. The net result of large elevations in intracellular calcium levels coupled with intracellular alkalosis due to NHE-mediated proton extrusion is tissue damage manifested by intracellular calcium overload, contracture, and depressed systolic function. Moreover, there is also evidence, par-

ticularly that based on NMR studies, to suggest that NHE activation during ischemia per se, that is prior to reperfusion, also contributes to both calcium and sodium overloading, providing yet another contributing factor to cell injury.<sup>18</sup>

#### *Attenuation of Cardiotoxic Effects of Ischemic Metabolites*

The above hypothesis suggests that the primary mechanism for NHE activation is intracellular acidosis. However, further NHE activation occurs because of direct stimulation by metabolites produced by the ischemic myocardium. For example, levels of endothelin-1 (ET-1), a potent NHE activator, are elevated in myocardial ischemia and may produce deleterious effects on the reperfused myocardium, inducing both diastolic and systolic abnormalities.<sup>19</sup> We have shown that the toxicity produced by ET-1 can be attenuated by NHE inhibition, suggesting an important role of the antiporter in mediating the detrimental effect of the peptide on the ischemic and reperfused myocardium.<sup>20</sup> NHE activation may also represent an important mechanism for arrhythmogenesis in the reperfused myocardium particularly under conditions of elevated catecholamine levels. For example, although  $\alpha_1$  adrenergic agonists enhance ventricular arrhythmias in the reperfused myocardium this effect can be markedly decreased by NHE inhibition.<sup>21</sup>

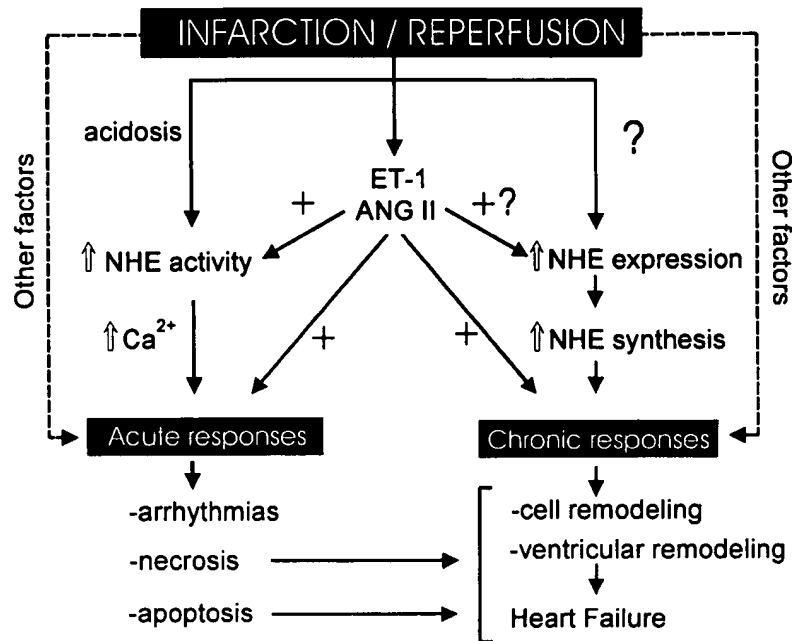
We have recently shown that lysophosphatidylcholine, one of the predominant tissue metabolites that accumulates rapidly in the ischemic myocardium, is a potent NHE activator in the cardiac cell and that the cardiotoxic effects of this amphiphile, at least at low concentrations, can be markedly attenuated by NHE inhibitors.<sup>22</sup> In addition, it appears that at least some of the direct toxic effects of hydrogen peroxide can be attenuated by NHE inhibition.<sup>23</sup> Moreover, we have recently demonstrated that the ability of very low, sub-toxic concentrations of hydrogen peroxide to compromise postischemic ventricular recovery can be attenuated by NHE inhibition.<sup>24</sup> Taken together, these observations open up the possibility that a variety of intracellular factors produced during ischemia may contribute to tissue dysfunction through NHE-dependent processes.

#### *Apoptosis*

There is now increasing evidence that apoptosis or "programmed cell death" is an important response of the myocardium to ischemia. Apoptosis is rapid, precedes cell necrosis, and appears to contribute the overall sequelae of cardiac injury.<sup>25-27</sup> We recently demonstrated that HOE 642 significantly attenuated the development of early apoptosis in hearts subjected to 30-min global ischemia with or without reperfusion.<sup>28</sup> Moreover, dietary cariporide inhibits reperfusion-associated apoptosis in the acutely infarcted myocardium (unpublished data). This is clearly an important area for further study, particularly, as discussed below, since apoptosis is emerging as an important contributor to the postinfarction remodeling process leading to heart failure.

### **EVIDENCE FOR A ROLE OF NHE IN POSTINFARCTION RESPONSES**

As evidenced from the preceding discussion, most studies on NHE involvement in heart disease have centered on acute responses. One study has shown that adding



**FIGURE 2.** Role of NHE in the acute responses to myocardial ischemia and reperfusion and potential consequences in terms of chronic postinfarction responses. See text for details.

amiloride to the drinking water of rats with infarcted myocardium resulted in a significant attenuation of ventricular remodeling.<sup>29</sup> Preliminary data from our laboratory demonstrate that dietary cariporide completely prevents ventricular fibrillation and mortality in these animals and reduces other forms of arrhythmias. Moreover, the degree of apoptosis was decreased as was the ability of infarction to upregulate NHE-1 expression. The potential benefits of the antiarrhythmic effects of cariporide are obvious in terms of early management following myocardial infarction. Apoptosis has emerged as a potentially critical factor in the response to infarction<sup>30</sup> and evolution to heart failure.<sup>31–33</sup> Thus, the fact that cariporide can inhibit this phenomenon<sup>28</sup> further reinforces its potential usefulness in postinfarction responses. Although the relevance is unclear at present, increased NHE-1 expression postinfarction is intriguing and suggests that NHE influences not only acute, but also chronic postinfarction responses, the latter occurring through an as-yet undefined mechanism of upregulated NHE-1 expression. Paracrine/autocrine regulation by ET-1 or angiotensin II may contribute to this process. These concepts are summarized in FIGURE 2.

### SUMMARY

There is now strong evidence that NHE activation in the ischemic and reperfused heart plays a major role in restoring pH<sub>i</sub>, which at the same time contributes to tissue

damage most likely via a number of complex mechanisms. This concept is supported by the fact that virtually all studies thus far reported have demonstrated cardioprotective effects of NHE inhibitors. In addition to its role in acute ischemia and reperfusion, it is likely that the antiporter will also be found to be of importance in other scenarios of ischemic injury, such as that involving long-term cardiac preservation. The beneficial effects of NHE inhibitors coupled with the likely low toxicity of these agents, particularly with respect to the novel isoform-specific inhibitors, provide promise for the development of new strategies for the protection of the ischemic myocardium as well for the heart subjected to reperfusion procedures.

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Invited review

# An overview of inhibitors of $\text{Na}^+/\text{H}^+$ exchanger

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## Abstract

The  $\text{Na}^+/\text{H}^+$  exchanger (NHE) is involved in intracellular pH homeostasis of many mammalian cell types. To date seven NHE isoforms (NHE1–NHE7) have been identified. NHE1 is the most predominant isoform expressed in heart where it contributes to cardiomyocyte pH homeostasis. Although the NHE activation is essential for the restoration of physiological pH, hyperactivation of NHE1 during ischemia–reperfusion episodes disrupts the intracellular ion balance, leading to cardiac dysfunction and damage. Beside its ability to inhibit a conductive  $\text{Na}^+$  channel and the  $\text{Na}^+/\text{Ca}^{++}$  exchanger, amiloride was the first drug described as NHE inhibitor. Double substitution of the nitrogen of the 5-amino group of amiloride gave DMA, EIPA, MIBA and HMA. Later, several acylguanidines were prepared to selectively inhibit NHE1. The replacement of the pyrazine ring of amiloride by a pyridine ring or by a phenyl increased the potency and the NHE selectivity. The simultaneous replacement of the pyrazine ring by a phenyl, of the 6-chloro by a sulfomethyl led to drugs such as HOE-694, cariporide, eniporide and BIIB-513 which also selectively inhibited NHE1. In the last decade several bicyclic guanidines were prepared: zoniporide, MS-31038, SM-20220, SM-20550, SMP-300, KB-R9032, BMS-284640, T-162559, TY-12533, S-3226 or SL-591227. Extensive pre-clinical studies indicated that NHE inhibitors afford substantial protection in different animal models of myocardial ischemia (MI) and reperfusion, but the results of clinical trials involving eniporide and cariporide were mixed.

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**Keywords:** Sodium-proton exchanger; NHE; Amiloride; Inhibitor; Ischemia–reperfusion

## 1. Introduction

The  $\text{Na}^+/\text{H}^+$  exchanger (NHE) is a protein that is expressed in many mammalian cell types. NHE is responsible for intracellular pH and cell volume regulation by extruding protons from, and taking up sodium ions into cells. To date seven isoforms (NHE1–NHE7) have been identified and cloned. NHE isoforms share ca. 20–60% amino acid identity and a molecular mass from 74 to 99 kDa (Table 1). NHE6 and NHE7 are localized to recycling endosomes and to the *trans*-Golgi network respectively, whereas the other isoforms (NHE1–5) are expressed in the cell membrane [1–3]. NHE isoforms are composed of 12 helical hydrophobic membrane-spanning segments, a N-terminal sequence and a highly hydrophilic C-terminal segment. The

segments M3–M12 share a high sequence homology among the various isoforms where M6 and M7 are most highly preserved (95% identity), suggesting that these domains are involved in the transport of  $\text{Na}^+$  and  $\text{H}^+$  across the membrane [1].

NHE1 is activable by growth factors and expressed in several cell types, mainly in mammalian cardiomyocytes, platelets and on the basolateral membrane of renal tubules [4,5]. NHE2 has been localized in the gastrointestinal system mainly in stomach, colon and small intestine, with lower levels in skeletal muscle and in selected nephron segments [6–8]. Some studies reported basolateral and other apical localization of NHE2 [1]. NHE3 is mainly expressed at high levels in colon, and small intestine, with significant levels also in kidney and stomach [9,10]. It contributes to sodium absorption by the brush-border membrane in intestinal or renal epithelia. NHE4 is highly abundant in stomach and also present at intermediate levels in small intestine and colon [11]. A lower concentration is found on the

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Table 1  
Isoforms of the  $\text{Na}^+/\text{H}^+$  exchanger

Isoform	Species	Structure	Localisation
NHE1	Human	815 91 kDa	Cardiomyocytes, platelets Basolateral membrane of several tissues
NHE2	Rat	813 91 kDa	Stomach, colon, small intestine, adrenal gland
	Rabbit	809 90 kDa	Kidney and intestinal epithelia cell
NHE3	Rat	831 93 kDa	Colon, small intestine, stomach apical membrane of epithelia (proximal tubule, intestine)
NHE4	Rat	81 kDa	Stomach, small intestine, colon, collecting tubule
NHE5	Human	896 99 kDa	Brain (hippocampus), spleen, testis, skeletal muscle
NHE6	Human	669 74 kDa	Brain, skeletal muscle, heart
NHE7	Human	725 80 kDa	Brain (putamen, occipital lobe), skeletal muscle Secretory tissues (stomach, prostate, pancreas, thyroid)

basolateral membrane of collecting tubule. Little is known about the role of NHE5 which is expressed predominantly in nonepithelial tissue such as brain (hippocampus, cortex) [12–14]. NHE5 has been identified at a lower level in spleen, testis and skeletal muscle. In contrary to NHE1–5, NHE6 is the first intracellular NHE. It has been identified on recycling endosomes but not in the inner membrane of mitochondria as primarily assessed [2,15]. NHE6 has been detected with highest abundance in brain and skeletal muscle, followed by heart and other tissues. NHE6 may regulate intravesicular pH and contribute to lysosomal biogenesis. Finally, NHE7 has been localized predominantly to the *trans*-Golgi network [3]. Its expression is ubiquitous but predominant in certain regions of brain (occipital lobe, putamen), in skeletal muscle, in stomach and in glands (pancreas, salivary-, thyroid- and mammary-gland ...).

NHE is working according to the  $\text{Na}^+$  and  $\text{H}^+$  gradients by exchanging an extracellular  $\text{Na}^+$  ( $\text{Na}_o^+$ ) against a intracellular  $\text{H}^+$  ( $\text{H}_i^+$ ) with a tightly coupled 1:1 stoichiometry. NHE1–3 and NHE5 exhibited a hyperbolic dependence on  $\text{Na}_o^+$  concentration ( $[\text{Na}^+]_o$ ) while NHE4 showed a sigmoidal dependence on  $[\text{Na}^+]_o$ . The affinity of these different NHE isoforms for  $[\text{Na}^+]_o$  is ranging between 5 and 50 mM (Table 2) [1]. Extracellular  $\text{Li}^+$  ( $\text{Li}_o^+$ ) and  $\text{H}^+$  ( $\text{H}_o^+$ ) competitively

inhibit  $\text{Na}_o^+$  influx by interacting at a single binding site of NHE1–3 and NHE5. In contrast to NHE2–3 and NHE5, extracellular  $\text{K}^+$  (1–100 mM) inhibits NHE1 ( $K_i = 180 \text{ mM}$ ) at high and nonphysiological concentrations [14,16]. Only NHE7 and NHE4 are able to mediate the influx of  $\text{K}^+$  or  $\text{Na}^+$  in exchange for  $\text{H}^+$  [3,17]. The decrease of intracellular pH enhanced the  $[\text{H}_i^+]/[\text{H}_o^+]$  gradient and activates NHE isoforms (Table 2) [18]. In absence of  $\text{Na}_o^+$ , NHE operates in a reverse mode by expelling  $\text{Na}^+$ .

NHE activity is regulated by several mechanisms [1]. Regulation of activity can be explained by direct phosphorylation of NHE by PKA and/or PKC [19]. NHE1 has been found to be constitutively phosphorylated in resting cells, and further phosphorylation is induced by phorbol esters, growth factors or phosphatase inhibitors [20]. The phosphorylation sites were detected on the distal part of the cytosolic C-tail. This NHE1 cytosolic tail contains also two calmodulin binding sites. Deletion of this segment constitutively stimulates NHE1 and mimics elevated intracellular  $[\text{Ca}^{2+}]$ . The unoccupied domain, able to bind calmodulin with high affinity, exerts an autoinhibitory effect [21]. The binding of a calcineurin homolog protein (CHP) to a NHE1 sequence located on the C-tail inhibits the NHE1 activity. CHP appears to be constitutively phosphorylated [22]. For NHE2, two proline-rich domains that resemble SH3-binding proteins have been identified in the C-tail region [23]. NHE activity is also regulated by GTP-binding proteins. Activated forms of  $\text{G}\alpha_q$ ,  $\text{G}\alpha_{12}$  and  $\text{G}\alpha_{13}$  activate NHE [24]. Recently, it has been evidenced that nitric oxide inhibited NHE3 activity via activation of soluble guanylate cyclase, resulting in an increase in intracellular cGMP levels and activation of protein kinase G [25].

Following intracellular acidosis, NHE activation is essential to restore physiological pH by  $\text{H}^+$  extruding. Nevertheless, an excessive stimulation of NHE results in an increase of intracellular  $\text{Na}^+$  concentration and a

Table 2  
Apparent affinity constants of rat NHE1–3 and human NHE5 isoforms for intra and extracellular monovalent cations

Cation	Apparent affinity constants ( $K_{0.5}$ ) <sup>a</sup>			
	NHE1	NHE2	NHE3	NHE5
$\text{Na}_o^+$ (mM)	10.0	50.0	4.7	18.6
$\text{Li}_o^+$ (mM)	3.4	2.2	2.6	0.32
$\text{K}_o^+$ (mM)	19.5	None	None	Slight inhibition
$\text{H}_o^+$ (pK)	7.00	7.90	7.00	8.13
$\text{H}_i^+$ (pK)	6.75	6.90	6.45	6.43

<sup>a</sup> Values from Refs. [14,16].

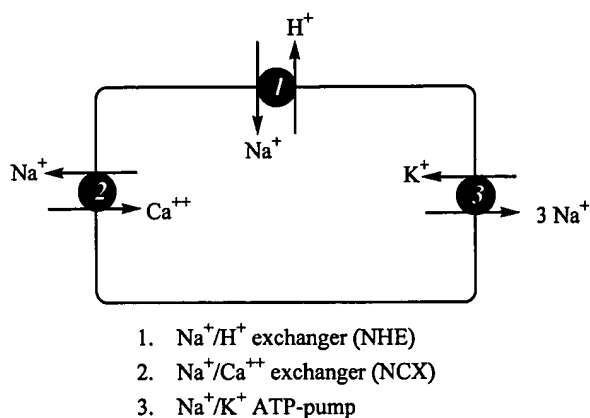


Fig. 1. Activation of NHE in ischemia–reperfusion: ischemia–reperfusion increase the  $\text{pH}_o/\text{pH}_i$  gradient which activate the NHE, enhance the activity of the  $\text{Na}^+/\text{K}^+$  ATP pump, and of the  $\text{Na}^+/\text{Ca}^{++}$  exchanger leading to intracellular  $\text{Ca}^{++}$  accumulation.

subsequent activation of  $\text{Na}^+/\text{K}^+$  ATPase, with a consecutive increase of energy consumption. The high intracellular  $\text{Na}^+$  level contributes to activate the sarcolemmal  $\text{Na}^+/\text{Ca}^{++}$  antiporter which lead to raised intracellular  $\text{Ca}^{++}$  (Fig. 1).

At the cardiac level, this cellular  $\text{Ca}^{++}$  overload subsequent to NHE-1 activation is involved in ischemic and reperfusion injuries like myocardial infarction activation, stunning and tissue necrosis [26]. It has been demonstrated that the activity of NHE-1 is also increased in red blood cells, platelets, leukocytes, and skeletal muscle cells from patients with essential hypertension [27–31]. In response to chronic or acute hypertension, NHE-3 is redistributed from the apical brush border of proximal tubules to intermicrovillar and endosomal stores [32]. Finally, insulin induced a significantly increased NHE1 activity in normal patients as compared to obese individual were erythrocytes are resistant to insulin [33].

## 2. NHE inhibitors

With the aim to attenuate the harmful consequences of excessive NHE activation, several inhibitors were developed with the primary goal to provide cardioprotective drugs by inhibiting the NHE1 subtype. Beside its ability to inhibit a conductive  $\text{Na}^+$  channel and the  $\text{Na}^+/\text{Ca}^{++}$  exchanger, amiloride, a  $\text{K}^+$ -sparing diuretic, was the first drug described as NHE inhibitor [34]. NHE1 and NHE2 are the most sensitive isoforms to amiloride inhibition whereas NHE3 and NHE4 are amiloride resistant isoforms [17] (Table 3). NHE5 is inhibited by amiloride at half concentration that was intermediate to those determined for NHE1 and NHE3 isoforms, but closer to the latter [14]. The latest isoform, NHE7 is insensitive to amiloride [3]. Cimetidine, harmaline and clonidine were also reported as weak and non-

specific NHE inhibitors [35]. To increase the potency and the selectivity of inhibitors towards the NHE isoforms, and particularly NHE1, several molecules derived from amiloride have been synthesized and investigated. Double substitution of the nitrogen of the 5-amino group gave DMA, EIPA, MIBA and HMA, the most studied pyrazines related to amiloride (Fig. 2). EIPA, HMA and DMA are much more effective than amiloride on each studied isoform and lost the inhibitory potency on  $\text{Na}^+$  channel and  $\text{Na}^+/\text{Ca}^{++}$  exchanger (Table 3). They are weak selective inhibitors of NHE1. The selectivity of EIPA is ranging as follow:  $\text{NHE1} > \text{NHE2} > \text{NHE5} > \text{NHE3}$ .

The pyrazine ring of amiloride was then replaced by a pyridine ring or by a phenyl. The phenyl counterpart of amiloride and the pyridine counterpart where the heterocyclic nitrogen was located in *meta* position of the acylguanidine were 54- and 36-times more active than amiloride on human platelet NHE1, respectively [36]. In the same experimental conditions, the pyridine derivative where the heterocyclic nitrogen was in *ortho* position of the acylguanidine was as active as amiloride [36]. Concomitantly to the replacement of the pyrazine ring of amiloride by a phenyl, the 6-chloro has been substituted by a sulfomethyl and the 2-amino has been deleted or replaced by a methyl group. Taken together, these modulations led to benzoylguanidines such as HOE-694 [37], cariporide [38], eniporide [39] and BIIB-513 [40] which completely lost the  $\text{Na}^+/\text{Ca}^{++}$  exchanger inhibitory potency as well as their ability to block  $\text{Na}^+$ -channels (Fig. 2). For each isoform investigated, HOE-694 is less active than EIPA but more selective towards NHE1 (Table 3). As compared to their inhibitory potency of NHE2, cariporide and eniporide are more NHE1-selective than EIPA. They are inactive on NHE3 and NHE5. As observed for the pyrazine derivatives, the substitution of the distal nitrogen of the acylguanidine moiety strongly decreased the NHE inhibitory potency. Later, several molecules based on a bicyclic template have been designed (Fig. 3). This bicyclic ring was a quinoline (zoniporide [41], MS-31038 [42]), an indole (SM-20220 [43], SM-20550 [44], SMP-300 [45]), a benzoxazinone (KB-R9032 [46]), a dihydrobenzofurane (BMS-284640 [47]), a tetrahydronaphthalene (T-162559 [48]), or a tetrahydrocycloheptapyridine (TY-12533 [49]). Excepted for T-162559, all these compounds bear an unsubstituted acylguanidine group. Miscellaneous compounds structurally far from amiloride were also prepared (S-3226 [50], SL-591227 [51]) (Fig. 3). Enzymatic studies assessed that zoniporide, BMS-284640, (S)-T-162559 and SL-591227 were selective NHE1 inhibitors as compared to other isoforms (Table 3), whereas SM-20220, SM-20550 and TY-12533 inhibit at least NHE1. Finally, S-3226 was the first NHE3 selective inhibitor.



Table 3  
Inhibitory potency of NHE inhibitors towards the different isoforms

Drug	Inhibitory potency (IC <sub>50</sub> or K <sub>i</sub> , in $\mu$ M) <sup>a</sup>					
	NHE1	NHE2	NHE3	NHE4	NHE5	NHE7
Amiloride	1–1.6* 5.3*	1.0**	> 100* 100–309*	813*	21	> 2000
EIPA	0.01*–0.02** 25.1*	0.08*–0.5**	2.4* 3.3*	> 10*	0.42 1.53	
HMA	0.013*		2.4*		0.37	
DMA	0.023*	0.25*	14*			
HOE-694	0.085*		640*		9.1	
Cariporide	0.03–3.4	4.3–62	1–> 100		> 30	
Eniporide	0.005–0.38	2–17	100–460		> 30	
Zoniporide	0.059	12	> 500*			
SM 20550	0.010*					
BMS-284640	0.009	1800	> 30		3.36	
T-162559 (S)	0.001	0.43	11			
T-162559 (R)	35	0.31	> 30			
TY-12533	0.017					
SL-591227	0.003	2.3				
S-3226	3.6	80**	0.02			
Harmaline	140*	330	1000*		940	
Cimetidine	26*	330	6200*		230	
	51*		> 1000*		> 1000*	
Clonidine	210*	42	620*		N.A.	

\* = from rat, \*\* = from rabbit. NA = not active. Values are from references [3,7,14,16,17,41,45,47–51,59].

<sup>a</sup> K<sub>i</sub> values are in italic.

The inhibitory potency of amiloride and some derivatives is reduced by high concentrations of Na<sup>+</sup>, suggesting that their cationic form bind to the external Na<sup>+</sup> binding site. The potency of NHE inhibitors is not only related to the chemical structure but also to the ionization of the guanidine function. At physiological pH (7.4), the acylguanidine of amiloride (pK<sub>a</sub> = 8.78) and the aminoguanidine of TY-162559 (pK<sub>a</sub> = 8.4) are completely protonated, and can interact with NHE under their cationic form [48,49]. During ischemia or reperfusion phase, the pH falls down to 6.2. In these conditions, drugs like cariporide (pK<sub>a</sub> = 6.28), TY-12533 (pK<sub>a</sub> = 6.93) or zoniporide (pK<sub>a</sub> = 7.2) are positively charged and then more efficient [41,49]. Indeed, cariporide and TY-12533 are more active at pH 6.2 than 6.7 (cariporide: IC<sub>50</sub> = 22 nM/120 nM; TY-12533: IC<sub>50</sub> = 17 nM/32 nM). This is also confirmed by the weak activity of the acylguanidine counterpart (pK<sub>a</sub> = 6.2; IC<sub>50</sub> = 210 nM) of the aminoguanidine TY-162559 (pK<sub>a</sub> = 8.4; IC<sub>50</sub> = 9 nM) [48].

### 3. Cardioprotective activity of NHE inhibitors

During myocardial ischemia, mitochondrial ATP synthesis ceases and glycolysis results in the depletion of ATP and in a decrease in intracellular pH which activates the NHE resulting in the extrusion of H<sup>+</sup> and the influx of Na<sup>+</sup>. Due to NHE activation and to Na<sup>+</sup>/

K<sup>+</sup> ATP-pump failure, the overload of intracellular Na<sup>+</sup> activates the Na<sup>+</sup>/Ca<sup>++</sup> exchanger which increases the cytosolic free calcium. The accumulation of intracellular Ca<sup>++</sup> contributes to cellular damage resulting in arrhythmias and myocardial stunning. With reperfusion, extracellular H<sup>+</sup> rapidly decreases increasing the intracellular to extracellular H<sup>+</sup> gradient. This large H<sup>+</sup> gradient activates NHE which enhances intracellular Na<sup>+</sup> and lead, through the Na<sup>+</sup>/Ca<sup>++</sup> exchanger, to accumulation of Ca<sup>++</sup> during reperfusion. This contributes to arrhythmia and myocardial contracture during the reperfusion period. As NHE1 is the predominant isoform in mammalian myocardium, the NHE1 inhibitors were investigated in several models of ischemia–reperfusion. Administered 15 min prior to a myocardial ischemia (1 h) induced by occlusion of the left anterior descending coronary artery in dogs, eniporide (0.75 mg kg<sup>−1</sup>, iv) significantly reduced the infarct size and the area at risk [52]. The reperfusion period was 3 h. In the same model, BIIB-513 (0.75 mg kg<sup>−1</sup>), administered either prior to ischemia or prior to reperfusion, reduced the infarct size, phase 1b arrhythmias and ventricular fibrillation induced by ischemia and reperfusion respectively [53]. Zoniporide (CP-597396), a highly soluble selective NHE1 inhibitor, was also investigated in a rabbit myocardial ischemia (0.5 h)–reperfusion (2 h) model. Infused 30 min before ischemia until the end of reperfusion phase, zoniporide reduced the infarct size to a greater extent than

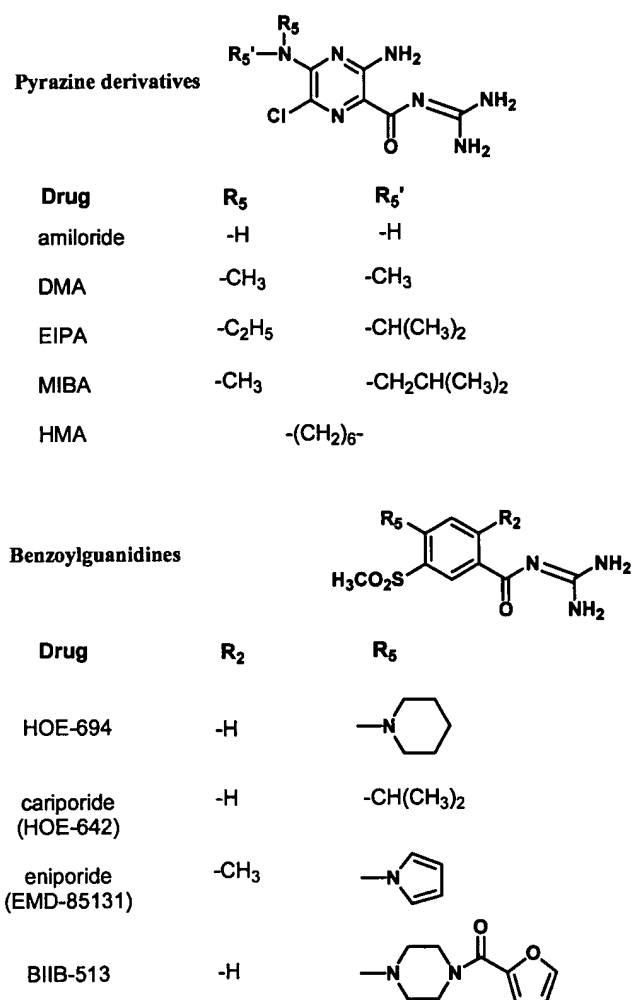


Fig. 2. Chemical structures of amiloride and of its pyrazine and phenyl derivatives.

eniporide or cariporide [54]. Furthermore, zoniporide did not cause any in vivo hemodynamic changes. In rabbit, SM-20550, an indole NHE-1 inhibitor which act on endothelial cells [55], 10 times more potent than EIPA, reduced the infarct size by ca. 30–70% in a dose-dependent manner (iv bolus 1.7–170  $\mu\text{g kg}^{-1}$  followed by iv infusion 2.8–280  $\mu\text{g kg}^{-1} \text{ h}^{-1}$ ) when administered prior to a myocardial ischemia–reperfusion (0.5–5 h) protocol [56]. Infused 10 min prior to the reperfusion period, the reduction was 20–40%. A similar experiment conducted in dogs showed that S-20550 (iv bolus 170  $\mu\text{g kg}^{-1}$  followed by iv infusion 280  $\mu\text{g kg}^{-1} \text{ h}^{-1}$ ) reduced the infarct size of 80 and 41% when administered 15 min prior to the occlusion of the left circumflex coronary artery (2 h) and to the reperfusion period (5 h), respectively [57]. Furthermore, SM-20550 suppressed ventricular fibrillation during both ischemia and reperfusion without affecting the size of the area at risk. SMP-300 inhibits NHE of rat myocytes with an  $\text{IC}_{50}$  of 6 nM and was therefore 16 times more potent than EIPA. SMP-300 (1  $\text{mg kg}^{-1}$ ), an orally active specific

NHE inhibitor, reduced rat myocardial infarct size after 40 min of coronary artery occlusion followed by 24 h of reperfusion [58]. The cardioprotective effect of (S)-T-162559, a specific NHE1 inhibitor 5 and 31 times more active than eniporide and cariporide, respectively [59], was studied in a rabbit model of ischemia–reperfusion (0.5–24 h) injury. Intravenously administered 5 min. prior to occlusion, (S)-T-162559 (0.03 and 0.1  $\text{mg kg}^{-1}$ ) reduced the myocardial area at risk by 36% [60]. The activity of TY-12533 was investigated in a rat model of myocardial ischemia–reperfusion (0.5–24 h), and compared to cariporide. Administered 5 and 10 min before the coronary snare occlusion and reperfusion respectively, TY-12533 and cariporide did not reduce the myocardial area at risk. The pre-occlusion treatment with TY-12533 and cariporide (0.1  $\text{mg kg}^{-1} \text{ iv}$ ) reduced the infarct size of 50 and 70%, respectively. After a post-occlusion treatment, only TY-12533 (0.1  $\text{mg kg}^{-1} \text{ iv}$ ) reduced the infarct size (44%). In dogs, TY-12533 (3  $\text{mg kg}^{-1} 10 \text{ min}^{-1}$ ) injected 10 min before or after a myocardial ischemia–reperfusion (0.25–2 h) did not affect reductions in regional myocardial wall thickening and blood flow during ischemia, but it improved these parameters after reperfusion [61]. SL-591227 is the first potent and NHE1-selective non-guanidine inhibitor. In rat following left coronary artery occlusion (7 min) and reperfusion (10 min), SL-591227 (10–100  $\mu\text{g kg}^{-1} \text{ min}^{-1} \text{ iv}$ ) inhibited ventricular tachycardia (71–100%) and fibrillation (75–87%) induced by ischemia and reperfusion respectively. In rabbit, SL-591227 (0.6  $\text{mg kg}^{-1} \text{ iv}$ ) reduced the myocardial area at risk (–58%) evoked by coronary occlusion and reperfusion (0.5–2 h) [51].

#### 4. Cerebroprotective activity of NHE inhibitors

Three NHE isoforms (NHE 1, 4 and 5) have been found in brain tissues and are expressed in neurons and glial cells. As observed for myocardial infarction, brain ischemia–reperfusion activates NHE which increases intracellular  $\text{Na}^+$ , cellular swelling and free  $\text{Ca}^{++}$  accumulation leading to cellular damage. SM-20220 inhibited recovery from acid load in cultured neurons and glial cells with an  $\text{IC}_{50}$  of 5 and 20 nM, respectively [43]. The effect of SM-20220, a specific NHE inhibitor structurally close to SM-20550 (Fig. 3), has been studied in Mongolian gerbil global cerebral ischemia [62]. Transient ischemia (30 min) was induced by clipping both common carotid arteries, and SM-20220 was intravenously infused (0.3 or 1  $\text{mg kg}^{-1}$ ) immediately after reperfusion. For each dosage, SM-20220 improved the neurological outcome (McGraw's score) for 24 h, and significantly reduced the mortality rate at 1  $\text{mg kg}^{-1}$ . SM-20220 and EIPA reduced free fatty acid from rat cerebral cortex during ischemia–reperfusion injury

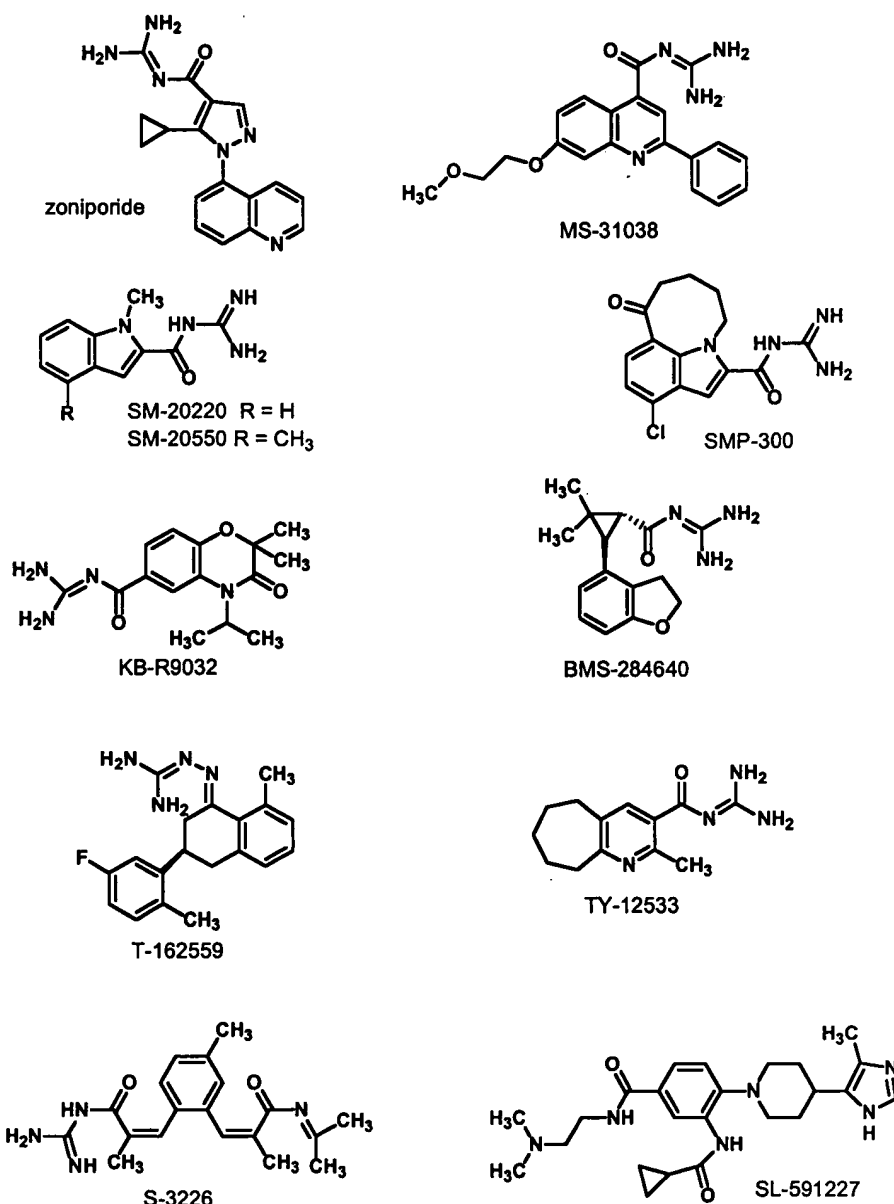


Fig. 3. Chemical structure of bicyclic NHE inhibitors.

[63,64]. These data indicate that these NHE inhibitors prevent the activation of phospholipases that occurs during reperfusion following a cerebral ischemia period. Finally, SM-20220, attenuated cerebral infarct volume, water content and neutrophil accumulation at 72 h after permanent occlusion of the rat middle cerebral artery [65].

## 5. Renal protection of NHE3 inhibitors

Acute renal failure is characterized by sudden loss of the kidney function due to ischemia, trauma, and/or nephrotoxic drugs. As described for heart and brain, renal ischemia activates NHE, and particularly the NHE3 isoform which is expressed at high level in

kidney. S-3226 is the first selective NHE3 inhibitor investigated in ischemia-induced acute renal failure in rats [66]. S-3226 (20 mg kg<sup>-1</sup>, iv) infused before or after global renal ischemia (40 min) enhanced the creatinine clearance and reduced the increase of plasma creatinine as compared to the control. On day 7 following renal ischemia, kidneys revealed pronounced reduction of tubular necrosis, dilatation, protein casts and cellular infiltration when treated with S-3226.

## 6. Clinical investigations of NHE inhibitors

Extensive pre-clinical studies indicated that NHE inhibitors afford substantial protection in animal models of myocardial ischemia (MI) and reperfusion, with a

high level of conformity between different investigators, species and models. To date, results of clinical investigations with cariporide and eniporide have been reported in patients with evolving myocardial infarction and in those at risk of myocardial infarction [67–70]. The effects of cariporide have been evaluated in patients subject to anterior MI who were expected to receive perfusion therapy by primary coronary angioplasty within 6 h of the onset of symptoms [68]. Patients ( $n = 100$ ) were randomized to receive placebo or cariporide (40 mg bolus iv) 10 min before reperfusion. Administration was completed within 4 h after the onset of symptoms. Cardiac enzymes and their isoforms (CK, CK-MB, LDH) were determined in blood samples taken before and after reperfusion (up to 72 h). Before treatment and at 3-week follow-up, contrast ventriculography was used to evaluate the left ventricular function. This study showed that the area under the curve of CK-MB release was reduced in the cariporide group as compared to the placebo group ( $P = 0.047$ ). The ejection fraction was higher in the cariporide group than in the placebo one, such that the change from baseline to follow-up was greater in the latter group ( $P = 0.045$ ). This study suggested that reperfusion injury could be a target for NHE inhibitors and these results warranted further clinical trials to confirm the therapeutic interest of NHE inhibitors. This led to the large-scale trial ESCAMI (Evaluation of the Safety and Cardioprotective Effects of Eniporide in Myocardial Infarction) [69]. This international, randomized, double-blind, placebo-controlled phase 2 trial enrolled 433 patients undergoing thrombolytic therapy or the percutaneous transluminal coronary angioplasty (PCTA) for acute ST-elevation MI to investigate the efficacy of eniporide on infarct size and clinical outcome. Eniporide was intravenously administered over 10 min. In patients receiving thrombolytic therapy the perfusion had to be completed at least 15 min after the start of treatment, while in patients subject to primary angioplasty, the infusion had to be completed at least 10 min prior to start PCTA. In stage 1 ( $n = 430$ ), four doses of eniporide were considered: 50, 100, 150 and 200 mg. This stage had a triple goal: to evaluate the primary efficacy end point determined by cumulative release of  $\alpha$ -hydroxybutyrate dehydrogenase ( $\alpha$ -HBDH) and of cardiac markers (CK-MB, troponin T and I), to select a subset of doses to be carried forward in stage 2, and to determine the number of patients to be enrolled for stage 2. Within the first 6 weeks, death, cardiogenic shock, heart failure, arrhythmias, major bleeding were considered as secondary end points. In stage 1, the administration of 100 and 150 mg eniporide resulted in smaller enzymatic infarct sizes, especially in angioplasty group. In contrast, in stage 2 there was no significant difference in the enzymatic infarct size between the three groups (placebo, 100 and 150 mg eniporide). Overall there was no effect of eniporide on

clinical outcome of secondary end points. However, a subgroup of patients ( $n = 322$ , 150 mg eniporide), in whom reperfusion was initiated more than 4 h after symptom onset, showed a significant reduction of heart failure symptoms when compared to the control group.

In the GUARDIAN (*Guard During Ischemia Against Necrosis*) trial ( $n = 11\,590$ ), the cardioprotective efficacy of cariporide was limited to high-risk patients who underwent coronary artery bypass graft (CABG) [67]. This trial failed to document benefit of cariporide over placebo on the primary end point of death or MI assessed after 36 days. Administered to the subpopulation of patients who underwent CABG, cariporide (120 mg three times a day) reduced of 25% the relative risk in the primary end point of death or MI. Further trials are warranted to confirm the cardioprotective benefit of NHE inhibitors in patients undergoing CABG surgery. The recently initiated EXPEDITION ( $\text{Na}^+/\text{H}^+$  Exchanger Inhibition to Prevent Coronary Events in Acute Cardiac Conditions) trial will test the hypothesis that NHE inhibition results in a reduction of MI in such patients [70].

These mixed results of clinical investigations with eniporide and cariporide contrast with the encouraging results obtained from preclinical studies, and the potential advantage of NHE inhibitors over other therapies claim further trials. Beside the cardioprotective effects of NHE1 inhibitors, the positive preclinical results obtained in the treatment of brain and renal ischemia reperfusion should be also verified in clinical trials [62–66].

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